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A secretion inhibitory signal transduction molecule on mast cells is another C-type lectin

(type 1 Fcε receptor/immunological stimulus)

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ABSTRACT **Secretion of inflammatory mediators by rat mast cells (line RBL-2H3) was earlier shown to be inhibited upon clustering a membrane glycoprotein by monoclonal antibody G63. This glycoprotein, named *mast* cell *function*-associated *antigen* (MAFA), was also shown to interfere with the coupling cascade of the type 1 Fcε receptor upstream to phospholipase $C_{\gamma 1}$ activation by protein-tyrosine kinases. Here we report that the MAFA is expressed as both a monomer and a homodimer. Expression cloning of its cDNA shows that it contains a single open reading frame, encoding a 188-amino acid-long type II integral membrane protein. The 114 C-terminal amino acids display sequence homology with the carbohydrate-binding domain of calcium-dependent animal lectins, many of which have immunological functions. The cytoplasmic tail of MAFA contains a YXXL (YSTL) motif, which is conserved among related C-type lectins and is an essential element in the immunoreceptor tyrosine-based activation motifs. Finally, changes in the MAFA tyrosyl- and seryl-phosphorylation levels are observed in response to monoclonal antibody G63 binding, antigenic stimulation, and a combination of both treatments.**

The type I receptor for IgE (FcεRI) is expressed on mast cells and basophils, as well as on Langerhans cells, monocytes, and eosinophils (1). FcεRI clustering on mast cells and basophils initiates a cascade of biochemical processes coupling it to the secretory responses of these cells. These processes include (*i*) activation of receptor-associated protein-tyrosine kinases (2) and phosphatases (3), causing transient tyrosine phosphorylation of several cellular proteins (4), (*ii*) an increase in phosphatidylinositide hydrolysis resulting from phospholipase Cγ1 activation, and (*iii*) a rise in the intracellular concentration of free calcium ions (1, 5). The final response to this stimulus is secretion of granule-stored mediators and the *de novo* synthesis and secretion of arachidonic acid metabolites and cytokines. Several membrane components different from the known FcεRI subunits have been identified by specific monoclonal antibodies (mAbs) (6–9) on the rat mucosal-type mast cells (line RBL-2H3) and shown to modulate FcεRI-mediated secretory response. G63, a mAb that binds a membrane glycoprotein, named *mast* cell *function*-associated *antigen* (MAFA), was shown to inhibit the FcεRI-induced signaling cascade upstream to phospholipase Cγ1 activation (i.e., before phosphatidylinositide hydrolysis and the transient rise in intracellular $[Ca^{2+}]$), the culminating degranulation (9), and secretion of interleukin 6 (M.D.G. and I.P., unpublished work). The mAb G63 inhibitory effect required MAFA clustering and was not due to interference with IgE–FcεRI interactions (9). Still, crosslinking of FcεRI–IgE complexes by multivalent antigen also led to coclustering of the MAFA with the aggregated FcεRI and to enhancement of its internalization (9, 10). The MAFA has been identified by immunoprecipitation with mAb G63 as a glycoprotein with a molecular mass of 28–40 kDa on reducing SDS/PAGE (9). We have now pursued its structural characterization¶ to resolve the basis for its inhibition of immunologically stimulated mast cell secretion.

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MATERIALS AND METHODS

Immunoprecipitation and N-Deglycosylation of MAFA. Cell preparation, surface radioiodination, and lysis were done as described (9). The MAFA was immunoprecipitated with mAb G63-coated beads (2–4 mg of IgG per g of agarose or Affi-Gel). Sedimented beads were either resuspended in deglycosylation buffer (see below) or boiled in SDS/PAGE sample buffer for elution and electrophoresis of bound MAFA. For cleavage of the N-linked oligosaccharide side chains, the sedimented beads were resuspended in 50 μl of 10 mM Tris·HCl, pH 7.0/0.1% SDS/0.5% 2-mercaptoethanol and boiled for 5 min. After cooling the mixture to room temperature, 1% Triton X-100, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride and *N*-glycosidase F at 4 units/ml (Boehringer Mannheim) were added, followed by incubation at 37°C for 18 hr with continuous gentle shaking. Then, the beads were boiled for 3 min in sample buffer, and the eluate was analyzed by SDS/PAGE.

Tryptic Peptide Mapping of the Lower and Higher Apparent-Mass Forms of the MAFA. Peptide mapping was done as described (11). Both forms of previously deglycosylated MAFA were localized by autoradiography on a polyacrylamide gel after nonreducing SDS/PAGE. The corresponding gel slices were then cut out and submitted to further radioiodination by the chloramine T method. The gel pieces were washed several times with 10% (vol/vol) aqueous methanol, dried, and rehydrated in 500 μl of trypsin at 50 μg/ml in 50 mM NH_4HCO_3 buffer, pH 8.0. Samples were incubated at 37°C for 12 hr with gentle agitation, after which the supernatants were removed, lyophilized, and analyzed on cellulose-coated plates by sequential high-voltage electrophoresis and chromatography (11). Finally, the plates were dried and autoradiographed.

RBL-2H3 cDNA Library Construction and Expression Cloning of the MAFA cDNA. Polyadenylylated RNA was prepared from RBL-2H3 cells and converted to double-stranded cDNA essentially as described (12). Size-fractionated cDNA >0.7 kb was pooled, ligated into the pcDNAI plasmid (Invitrogen), and electroporated into *Escherichia coli* MC1061/P3 (12). Batches of ≈10,000 colonies of transformed bacteria were prepared. Plasmid DNA was isolated from each pool and used to transfect COS-7 cells with the DEAE-dextran/chloroquine method (13). A ligand-binding assay of

Abbreviations: MAFA, mast cell function-associated antigen; mAb, monoclonal antibody; FcεRI and FcεRII, type I and type II receptor, respectively, for IgE; *P*-Ser, phosphoserine; *P*-Tyr, phosphotyrosine.

‡M.T. and I.P. contributed equally to this work.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. X79812).

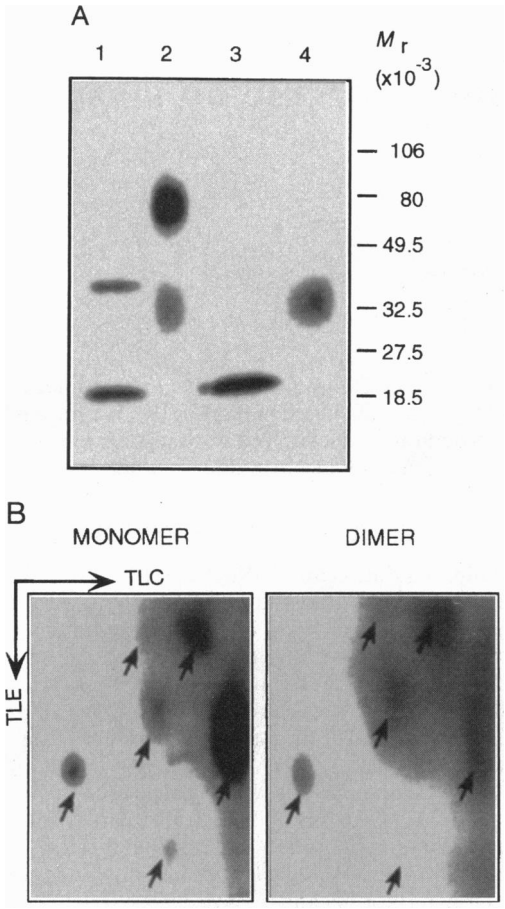

FIG. 1. (*A*) Electrophoretic analysis of native and N-deglycosylated MAFA. A lysate of 10^8 surface-radioiodinated RBL-2H3 cells was incubated with mAb G63-coated beads, which were then washed and incubated with *N*-glycosidase F (lanes 1 and 3) or with buffer alone (lanes 2 and 4). Immunoprecipitates were eluted by boiling in SDS/PAGE sample buffer and analyzed on a 10–17.5% polyacrylamide gel under reducing (lanes 3 and 4) or nonreducing conditions (lanes 1 and 2). The gel was then dried and autoradiographed. (*B*) Two-dimensional tryptic peptide mapping of the monomeric and dimeric forms of *N*-deglycosylated MAFA. The 18- and 40-kDa forms were separated by SDS/PAGE as described above, localized by autoradiography, and excised. The two gel pieces were then submitted in parallel to further radioiodination followed by tryptic proteolysis, and the eluted fragments were then separated by electrophoresis in one dimension and chromatography in the other. An autoradiogram of a plate is presented. Arrows indicate similar spots. TLE, thin layer electrophoresis.

the transfected monolayers was done with ^{125}I-labeled mAb G63 (chloramine T method; 6700 cpm/fmol, 1 nM). Autoradiographic detection of transfected cells was essentially as described (14). Positive bacterial pools (i.e., those yielding plasmid DNA capable of driving expression of the mAb G63 epitope by transfected COS-7) were then subdivided into smaller pools. The procedure was repeated until the final isolation of positive clones.

Endoproteinase Lys-C Digestion of MAFA. The deglycosylated MAFA was digested with endoproteinase Lys-C (Boehringer Mannheim) essentially as described (15). mAb G63 immunoprecipitates of surface-radiolabeled RBL-2H3 cell lysates were deglycosylated, submitted to reducing SDS/PAGE, and electrotransferred to nitrocellulose. Bands of interest were then visualized by autoradiography, and the corresponding nitrocellulose strips were excised and incubated with 0.5 unit of endoproteinase Lys-C in 50 μl at 37°C for 24 hr. The peptides were then eluted by sonication and analyzed by SDS/Tricine/PAGE using a 16.5% T/6% C separating gel

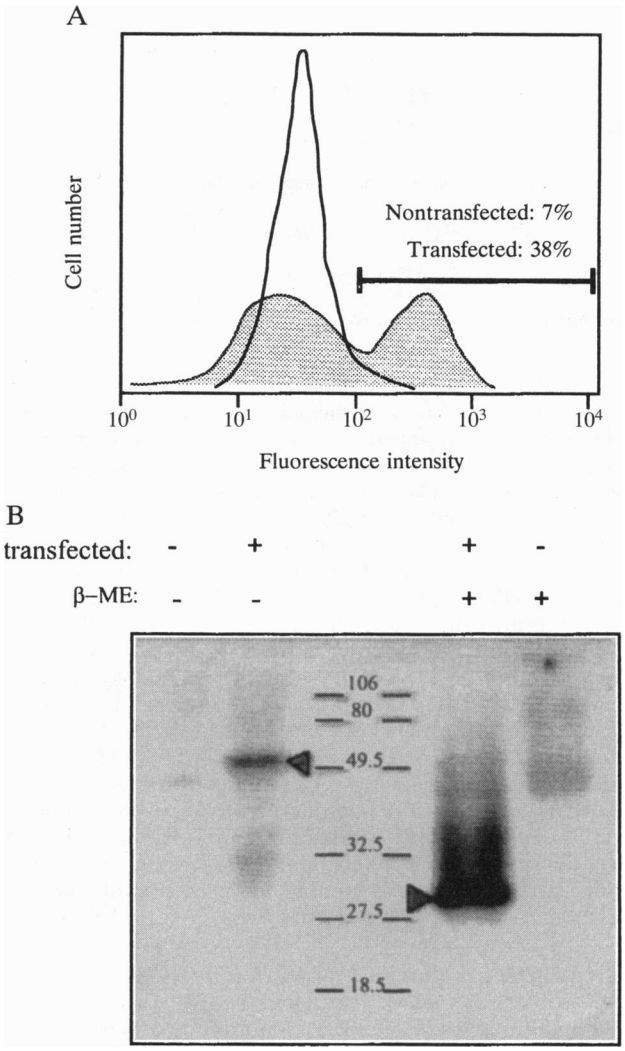

FIG. 2. (*A*) Flow cytometric analysis of COS-7 cells transfected with the cloned MAFA cDNA and stained with mAb G63. Transfected COS-7 cells (10^6) were incubated for 30 min at room temperature in the presence (shadowed) or absence (blank) of biotinylated mAb G63 at 0.5 mg/ml, washed, and further incubated for another 30 min at 37°C in the presence of phycoerythrin-labeled streptavidin at 25 μg/ml. Five thousand cells of each sample were analyzed by a Becton Dickinson FacScan flow cytometer. The derived histograms are overlaid; the staining percentage is also indicated. (*B*) Immunoprecipitation and SDS/PAGE analysis of the protein encoded by the cloned cDNA. COS-7 cells (10^7), untransfected or transfected with the cloned cDNA, were surface-radioiodinated, and the derived lysates were incubated with mAb G63-coated beads. Immunoprecipitates were eluted by boiling in SDS/PAGE sample buffer and separated on a 12.5% polyacrylamide gel under reducing or nonreducing conditions. BME, 2-mercaptoethanol. The gel was then dried and autoradiographed. Arrowheads indicate relevant bands.

(16) that was dried and autoradiographed (T denotes the total concentration of both acrylamide and bisacrylamide monomers, and C denotes the concentration of the crosslinker).

^{32}P Labeling and Phospho Amino Acid Analysis. RBL-2H3 cells (5×10^6) were plated per 100-mm tissue culture dish and cultured for 24 hr. The monolayers were then washed twice with phosphate-free Dulbecco's minimal Eagle's medium (DMEM) and starved by a 3-hr incubation in phosphate-free DMEM/2% fetal calf serum (previously dialyzed against deionized water)/50 mM Hepes, pH 7.4 (phosphate-free medium). Cells were washed again and further incubated for 3 hr with 3.5 ml of phosphate-free medium supplemented with 750 μCi (1 Ci = 37 GBq) of [^{32}P]P_i (Rotem Industries, Beer-Sheva,

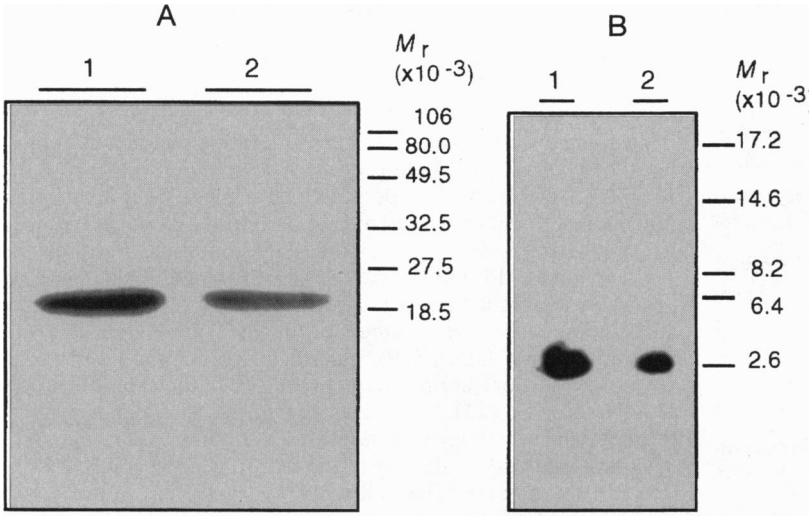

FIG. 3. (*A*) N-Deglycosylation of the protein encoded by the cloned cDNA. Cloned cDNA-transfected COS-7 cells (10^7) and RBL-2H3 cells (10^7) were surface-radioiodinated, and the derived lysates were separately incubated with mAb G63-coated beads, followed by washing and incubation with *N*-glycosidase F, as described. The immunoprecipitates derived from each sample were then subjected to reducing SDS/15% PAGE and electrotransferred to a nitrocellulose membrane, which was then autoradiographed. (*B*) Endoproteinase Lys-C digestion of both the protein encoded by the cloned cDNA and the RBL-2H3-derived MAFA. The nitrocellulose strips containing the 20-kDa polypeptide cores shown in *A* were excised and incubated with endoproteinase Lys-C. The proteolysis products derived from each sample were then eluted and subjected to electrophoresis in a Tricine/SDS/polyacrylamide gel that was dried and autoradiographed.

Israel). Samples scheduled for antigenic stimulation were primed by the addition of 10 nM 2,4-dinitrophenyl-specific, IgE-class mAb (SPE-49) simultaneously with the P_i and stimulated after the phosphate-incorporation period by the addition of $(2,4\text{-dinitrophenyl})_{11}$-bovine serum albumin at 100 ng/ml for 2 min. Where indicated, 10 nM mAb G63 was added to the cells 10 min before lysis or antigen stimulation. The above treatments were stopped by two fast washings with ice-cold phosphate-buffered saline (PBS) and the immediate addition of 1 ml of the above-described lysis buffer/100 mM NaF/2 mM $NaVO_4$/10 mM sodium pyrophosphate (17). The cell lysates were transferred to 1.5-ml test tubes and incubated with 20 μl of mAb G63-coated agarose beads and 4 μl of protein A–Affi-Gel (Bio-Rad) for 3 hr at 4°C with gentle rocking. After sedimenting, the beads were incubated with *N*-glycosidase F as described above. Eluates were subjected to SDS/15% PAGE. The proteins were then electrotransferred to a poly(vinylidene difluoride) membrane and visualized by autoradiography (18). The MAFA-containing poly(vinylidene difluoride) strips were excised and heated to 110°C in 200 μl of 6 M HCl for 1 hr in sealed tubes. The supernatants were then transferred to fresh test tubes and dried by evaporation. Identical quantities of the hydrolysates were dissolved in 5 μl of glacial acetic acid/pyridine/water, 5:0.5:94.5 (vol/vol/vol), pH 3.5. The phospho amino acids were separated by electrophoresis on cellulose-coated glass plates in the above buffer at 1 kV for 25 min. The plates were then air-dried, and internal phospho amino acid standards (1 μg each) were visualized by spraying with 0.2% (wt/vol) ninhydrin/ethanol. The radioactive phospho amino acids were then detected by autoradiography, and their intensity was read by densitometry.

RESULTS AND DISCUSSION

The MAFA Polypeptide Core Is 20 kDa. Immunoprecipitates of mAb G63 from surface-radioiodinated RBL-2H3 cells were resolved (9) by nonreducing SDS/PAGE as two broad radioactive bands (28–40 kDa and 60–82 kDa) (Fig. 1*A*, lane 2). Under reducing conditions, only the lower band was observed (Fig. 1*A*, lane 4). To assess the N-linked-oligosaccharide content of the MAFA, mAb G63 bead–immunoprecipitate, obtained as before from surface-iodinated cells, was incubated with *N*-glycosidase F and eluted by boiling in sample buffer; the eluate was then analyzed by SDS/PAGE. Under nonreducing conditions, two considerably narrower bands (18.5 and 40 kDa) were resolved (Fig. 1*A*, lane 1). Hence, N-linked oligosaccharides account for up to half of the apparent molecular mass of the MAFA. The approximate 1:2 apparent mass ratio of the two deglycosylated species suggested that the heavier one is a homodimer. Under reducing conditions, only the low-molecular-mass form of the MAFA was observed, although with a slightly reduced mobility (20 kDa, Fig. 1*A*, lane 3).

Subunit Composition of the MAFA. To examine whether the isolated higher-molecular-mass species is, indeed, a homodimer and exclude the possibility that it is a heterodimer with a subunit that undergoes no cell-surface iodination, peptide maps were prepared from tryptic proteolysis fragments of both N-deglycosylated 18.5- and 40-kDa forms. After proteolysis with trypsin, the peptides eluted from the gel pieces were loaded on cellulose-coated glass plates and separated in the first dimension by electrophoresis and by chromatography in the second dimension (Fig. 1*B*). The marked similarity of the peptide maps derived from both forms of the MAFA indicated that the 40-kDa species is, indeed, a homodimer and that the MAFA is isolated from RBL-2H3 cell lysates both as a monomer and a disulfide-linked homodimer.

Cloning and Expression of the MAFA cDNA. A eukaryotic cDNA expression library was constructed by using polyadenylylated mRNA isolated from RBL-2H3 cells, which constitutively express the MAFA (9). Sixty-five pools of 10,000 cDNA clones each were transfected into COS-7 cells, which were screened for ^{125}I-labeled mAb G63-binding cells, as described above. One of the 65 cDNA pools conferred on the transfected cells the ability to bind the radioiodinated mAb G63. Complete inhibition in the presence of a 100-fold excess of unlabeled mAb G63 established binding specificity. Moreover, the binding was unaffected by a similar excess of an isotype-matched (IgG1) antibody, excluding possible interactions via Fcγ receptors. Subpooling and further screening of the positive pool led to the isolation of a positive clone. Expression of the cloned cDNA by transfected COS-7 cells was analyzed by flow cytometry after sequential incubation of the transfected cells with biotin-labeled mAb G63 and phycoerythrin-labeled streptavidin. Thirty-eight percent of the transfected COS-7 cells stained with mAb G63 (Fig. 2*A*), in accordance with the independently determined transfection efficiency (10–50%). To determine the apparent molecular mass of the recombinant protein encoded by the cloned cDNA, monolayers of 10^7 transfected and control, untransfected COS-7 cells were surface ^{125}I-labeled and lysed. Immunoprecipitation with mAb G63-coated beads followed by elution, SDS/PAGE analysis, and autoradiography revealed a single labeled band with an apparent molecular mass of 58 kDa under nonreducing conditions and 28 kDa under reducing conditions (Fig. 2*B*). The slight difference between apparent masses of the proteins isolated from the RBL-2H3 and transfected cells might be due to differences in posttranslational modifications. Therefore, the apparent molecular masses of the respective

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   1 CACCCTGCTTACTGTCGTCACTCCCTGCTGAGTGTGAAGGGCGTTGGGTGGAGATGGCCG
   1                                                   M  A  D

  61 ACAACTCTATCTACTCAACATTAGAGCTGCCTGCTGCACCTCGAGTCCAAGATGACTCCA
   4  N  S  I  Y  S  T  L  E  L  P  A  A  P  R  V  Q  D  D  S  R

 121 GATGGAAGGTCAAAGCTGTCTTACACCGACCCTGTGTTTCCTACCTTGTGATGGTGGCTT
  24  W  K  V  K  A  V  L  H  R  P  C  V  S  Y  L  V  M  V  A  L

 181 TGGGGCTTTTGACTGTGATTCTCATGAGTCTACTGTTGTACCAACGGACTCTGTGCTGTG
  44  G  L  L  T  V  I  L  M  S  L  L  L  Y  Q  R  T  L  C  C  G

 241 GCTCCAAGGGCTTTATGTGTTCCCAGTGCTCCAGGTGCCCCAACCTCTGGATGAGGAACG
  64  S  K  G  F  M  C  S  Q  C  S  R  C  P  N  L  W  M  R  N  G

 301 GGAGCCACTGTTACTACTTCTCAATGGAGAAAAGGGACTGGAACTCTAGTCTGAAGTTCT
  84  S  H  C  Y  Y  F  S  M  E  K  R  D  W  N  S  S  L  K  F  C

 361 GTGCAGACAAAGGCTCGCATCTCCTTACATTTCCGGACAACCAGGGAGTGAACCTGTTCC
 104  A  D  K  G  S  H  L  L  T  F  P  D  N  Q  G  V  N  L  F  Q

 421 AGGAGTATGTGGGCGAGGACTTTTACTGGATTGGCTTGAGGGACATCGATGGCTGGAGGT
 124  E  Y  V  G  E  D  F  Y  W  I  G  L  R  D  I  D  G  W  R  W

 481 GGGAAGATGGCCCAGCTCTCAGCTTAAGCATTCTCTCTAACAGCGTGGTACAGAAGTGTG
 144  E  D  G  P  A  L  S  L  S  I  L  S  N  S  V  V  Q  K  C  G

 541 GCACCATCCACAGGTGTGGCCTCCACGCCTCCAGTTGTGAGGTTGCTTTGCAGTGGATCT
 164  T  I  H  R  C  G  L  H  A  S  S  C  E  V  A  L  Q  W  I  C

 601 GTGAGAAGGTCCTGCCCTGAAGGATTCCACTGTGTCCCAAGCCTCAGATCTGCCACATGT
 184  E  K  V  L  P  *

 661 CTTCAAAAAGAGGGAATGGGCATGGGGAACCTCTGTTCACAAAGGTGTCTTTAGCAAATG
 721 CCAAACCTGTTATGATATGCCATTAGACAGGCGTTAGCATTCCTTCCTGGGAGCTGGCAT
 781 TTTTCAACTGGGCTTTCTCAGTCATGTTAGCCATTTAAAGCCTAAATCTGGGCAAATGAA
 841 ATAGATAAAATTTATTTTGATGGCTCTTACTGCACAAACTCACCCTGGCTTTCTCATCCC
 901 ATACTCTGCCATATCTATCAAAGATATGTGCAAAACTATTCATCTGCAGAAGAACCCCCA
 961 CCACGGTCAATAACACATTACATAGACATCGAATAGAGACAGAAAAGCAAACACCTCCTG
1021 TTCTCACTCCTGCTTGGAAGCTGAAGTAGCTCAAGCCTGAGGTGTAGGGAGAAGTGCAGT
1081 GGTTACCAGAGTCCAGGAGACTGAAGGGATGGTAGAGGTTGGTTAATGGTTTGGCTGGTG
1141 TGGGGTGACCATCATGATTAATGATTGTTGTATGTTTGCCAATATGTTGTGAACTTCCGG
1201 ATAGCGAGGTGGAAGGACCGTGGGTGTTACCAAATGCCTGCAGGAGAGATGTGCTGAGAA
1261 CCCTGACTGGATGATTTCCACACACATTGAAATATCACACTGTGCCCCATAAATGTGTAC
1321 AATCATTATCTATCCCTAATTTCCCTAAAAATTAAAGAAGTCCCAATTAAAATAAAAAAT
1381 ACCTTTCTGCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
1421 AAAAAAAAAAAAAAAAAAAA
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FIG. 4. Sequence of cloned MAFA cDNA. The nucleotide and deduced amino acid (one-letter code) sequences of the MAFA are shown. Bases and amino acids are numbered at left. The predicted transmembrane domain is underlined, the putative N-glycosylation sites are boxed, and the cysteines are circled. The TGA stop codon is marked with a star, and the polyadenylylation signal is overlined.

N-deglycosylated polypeptide chains were compared. Both protein samples were found (Fig. 3*A*) to have a 20-kDa polypeptide core, supporting the hypothesis that the cloned protein is MAFA. To further substantiate this point, both 20-kDa polypeptides (shown in Fig. 3*A*) were digested in parallel by endoproteinase Lys-C. Analysis of the resulting peptide fragments by Tricine/SDS/PAGE and autoradiography revealed, in both cases, a single radioactively labeled peptide of 2.6 kDa (Fig. 3*B*). Taken together, these results indicate that the cloned cDNA sequence, indeed, codes for the MAFA.

Determination of the MAFA cDNA Sequence. The complete nucleotide sequence of the MAFA cDNA is presented in Fig. 4. An open reading frame deduced from the nucleotide sequence starts at nt 54 with a methionine codon and ends at nt 617 before a TGA stop codon. A search for homology to the MAFA cDNA nucleotide sequence did not yield any significant result. However, when the search was done at the deduced amino acid level, marked homology was found between the C-terminal 114 amino acids of the MAFA and the carbohydrate-recognition domain of several type II integral membrane proteins. These are members of the Ca^{2+}-dependent (C type) animal lectin family (19), including two hepatic lectins—the murine asialoglycoprotein receptor 2 (20) and the rat Kupffer cell receptor (21). The other C-type lectins displaying high sequence homology with the MAFA are involved in immunological functions: the type II Fcε receptor (FcεRII/CD23) (22), the natural killer cell antigens NKR-P1 (23) and Ly-49 (24), the T-cell early-activation antigen CD69 (25–27), and the B-cell differentiation antigen CD72 (28). Amino acid sequence alignment of the carbohydrate-recognition domains of the MAFA and several of the latter lectins shows conservation of 15 residues (6 cysteines, 5 tryptophans, 2 glycines, and 2 leucines) interspersed within this 114- to 129-amino acid-long domain (Fig. 5). Furthermore, the WIGL and CYYF motifs are highly conserved in all these proteins (29). The disulfide bonds linking the conserved cysteines (30) create subdomains probably with similar length in all these lectins, thereby yielding a common carbohydrate-recognition domain folding pattern. Which of the remaining four cysteinyl residues of the MAFA provides its covalent dimerization has yet to be determined. As with other type II integral membrane proteins, no signal sequence could be identified.

The hydropathy plot suggests only a single 21- to 23-amino acid-long transmembrane domain (Fig. 4, underlining) located 35 residues from the N terminus. A search for motifs predicts two putative N-glycosylation sites located at the C-terminal side of the transmembrane domain. These observations, taken together with the marked decrease in mAb G63 binding to the deglycosylated MAFA (M.D.G. and I.P., unpublished work), strongly support the proposed MAFA orientation as a type II membrane protein.

The deduced cytoplasmic-domain sequence of the MAFA exhibits a putative casein kinase II phosphorylation site on Ser-8. This serine/threonine kinase is independent of cyclic nucleotides and of calcium and participates in cell-cycle-progression control of several cell types, including B lymphocytes (31). Significantly, Ser-8 is part of a YSTL sequence related to YXXL/I motifs present in the cytoplasmic domains of several other C-type animal lectins: in chicken hepatic lectin YVLL, the human asialoglycoprotein receptor YEDL, the human FcεRII YSEI, the human CD72 YADL, the mouse

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r-MAFA (188)   75  C PNL W MRNGSH C YYFSMEKRD W NSSLKF C ADKGSH L LTFPDNQGVNLFQEYVGEDF..Y
m-Fce2 (331)  186  C PKN W LHFQQK C YYFGKGSKQ W IQARFA C SDLQGR L VSIHSQKEQDFLMQHINKKDS..
h-CD69 (199)   85  C SED W VGYQRK C YFISTVKRS W TSAQNA C SEHGAT L AVIDSEKDMNFLKRYAGREE..H
m-NK11 (227)   94  C PQD W LSHRDK C FHVSQVSNT W EEGLVD C DGKGAT L MLIQDQEELRFLLDSIKEKYNSF
m-LECI (301)  170  C PVN W VEFGGS C YWFSRDGLT W AEADQY C QLENAH L LVINSREEQDFVVKHRSQFHI..

W I GL R.DIDG..WR W ED G PALSLSI.....LSNSV...VQK C GTIHRCGLHA.SS C EV.ALQ W I C EKVLP  188
W I GL QDLNMEGEFV W SD G S..PVGYSNWNPGEPNN.....GGQGED C VMMRGSGQWNDAF C RSYLDA W V C EQLAT  310
W V GL K.KEPGHPWK W SN G KEFNNW.....FNVTG...SDK C VFLKNTEVSS.ME C EK.NLY W I C NKPYK  199
W I GL RYTLPDMNWK W IN G STLNSDV.....LKITGDTENDS C AAISGDKVTFE.S C .NSDNR W I C QKELY  215
W I GL TD..RDGSWK W VD G TDYRSNYRNWAFTQPDNWQGHEQGGGED C AEILSDGHWNDNF C QQ.VNR W V C EKRRN  298
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FIG. 5. Sequence homology between the MAFA and members of the C-type lectin family. The MAFA carboxyl-terminal 114 amino acids are aligned with the carbohydrate-recognition domain of selected C-type lectins that share the highest degree of homology. Fully conserved residues are boxed, and motifs CYYF and WIGL are shadowed. The first and last amino acids of each displayed sequence are numbered in boldface type, and the total length of the respective polypeptide is in parentheses. m-LECI, mouse asialoglycoprotein receptor 2; m-Fce2, mouse FcεRII; h-CD69, human CD69; m-NK11, mouse natural killer cell antigen NKR-P1. Sequences were aligned with the PILEUP program in the Genetics Computer Group sequence-analysis software.

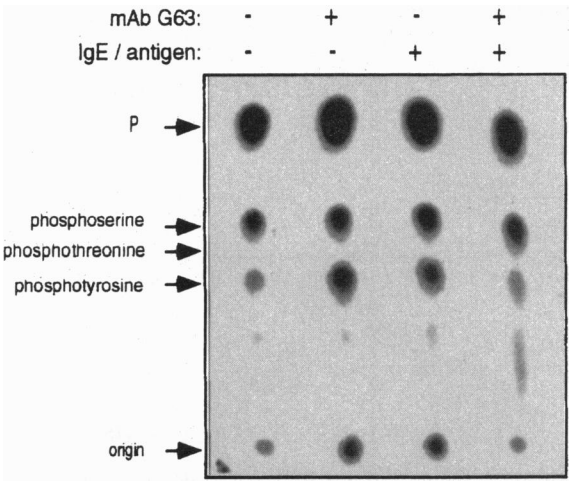

FIG. 6. Phospho amino acid analysis of ^{32}P-labeled MAFA. The poly(vinylidene difluoride) strips containing similar amounts (by radioactivity) of biosynthetically ^{32}P-labeled MAFA were excised, washed, and heated to 110°C in 6 M HCl for 1 hr. The solvent was then removed by evaporation, and the hydrolysates were redissolved, loaded on cellulose-coated glass plates, and separated by electrophoresis. One microgram of nonradioactive *P*-Ser, phosphothreonine, and *P*-Tyr was used as internal standard and visualized by ninhydrin staining. An autoradiogram of the chromatographed phospho amino acids is presented.

Ly49B YTTL, the rat NKR-P1 YLSL, and the mouse NKR-P1 YFGL. YXXL/I motifs are a central element of the immunoreceptor tyrosine-based activation motifs present (in reversed order to that appearing in type II proteins) in all multichain immune recognition receptors—e.g., in the T-cell receptor ζ chain and CD3 chains (32, 33). The tyrosyl of a related motif (YSLL) in the cytoplasmic tails of mouse FcγRIIb1 and FcγRIIb2 was shown to be essential for FcγRIIb2-mediated endocytosis and phagocytosis by mast cells (34). An identical motif was found in FcγRII of rat (35). The YSLL motif in human FcγRIIB has been shown to be responsible for abrogation of B-cell activation triggered by membrane immunoglobulin clustering. Coclustering the FcγRIIB with membrane immunoglobulin results in phosphorylation of the YSLL tyrosine, which, in turn, could lead to recruitment of Src homology 2 domain containing enzymes (e.g., phosphatases) to counteract cellular activation (36). By analogy, it would be of interest to determine whether the MAFA YSTL sequence is a target for any Src homology 2 domain-containing protein.

Phospho Amino Acid Analysis of MAFA. To determine whether MAFA undergoes phosphorylation and how FcεRI and the MAFA clustering affect it, metabolic labeling of RBL-2H3 cells with ^{32}P P_i was undertaken, and the results established that the MAFA is, indeed, phosphorylated (data not shown). Phospho amino acid analysis of the MAFA was done on samples derived from resting and FcεRI-stimulated cells, both with and without MAFA clustering by mAb G63. No ^{32}P-labeled phosphothreonine could be seen in any of the samples, irrespective of cell treatment. In contrast, labeled phosphoserine (*P*-Ser) and phosphotyrosine (*P*-Tyr) were found in samples derived from resting cells (Fig. 6), suggesting that the MAFA is constitutively phosphorylated on both its cytoplasmic tyrosine (probably Tyr-7 of the YSTL motif) and on one or more of its three cytoplasmic seryl residues. Densitometry of the autoradiogram was done, and the results presented are intensities of *P*-Tyr and *P*-Ser, both normalized to that of P_i in the respective lane: While resting cells samples yielded values for *P*-Tyr of 0.12 and for *P*-Ser of 0.29, mAb G63 binding enhanced the *P*-Tyr value to 0.25, leaving the *P*-Ser value essentially the same (0.22). FcεRI clustering similarly increased *P*-Tyr to 0.23 and *P*-Ser to 0.31. FcεRI clustering in the presence of mAb G63 lowered the *P*-Tyr value to 0.16 and the *P*-Ser value remained unchanged (0.29). Further studies are needed to attain a clearer correlation between the latter data and the cellular response. Such studies will allow speculation as to whether the inhibitory effect of the MAFA on RBL-2H3 cellular response to antigenic stimulation is exerted via the YSTL motif. Interactions between YXXL motifs and Src homology 2 domains are central to coupling cascades (32, 33, 36). Clustering the MAFA by mAb G63 may provide the avidity required for recruitment of Src homology domain 2-containing enzymes, such as protein-tyrosine phosphatases, which would interfere with the FcεRI coupling cascade as observed upstream to phospholipase $C_{\gamma 1}$ activation.

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